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Unfolding of Ribonuclease A on Silica Nanoparticle Surfaces

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ABSTRACT

This paper reports on the unfolding behavior of ribonuclease A (RNase A) on silica nanoparticle surfaces and quantitively demonstrates that nanoscale size and surface curvature play key roles in influencing the stability of adsorbed proteins. Urea denaturation analyses showed that the thermodynamic stability of RNase A decreased upon adsorption onto the nanoparticles, with greater decrease on larger nanoparticles. The stability changes of RNase A correlate well with the changes in the protein–nanoparticle interactions, which increase as the surface contact area and surface charge interaction increases. This study, therefore, provides fundamental information on the effect of nanoscale surfaces on protein structure and function.

Understanding the interaction of biomolecules with nanoscale surfaces is fundamentally important to the design and creation of hierarchical material assemblies and functional devices using biomolecules and to elucidate potential health effects, either positive or negative, of nanoscale materials at the molecular level. Proteins are known to undergo changes to their structures and stabilities upon adsorption onto macroscopic solid surfaces.[1,2] Such changes are strongly dependent on both the nature of the adsorbed proteins and the physicochemical characteristics of the solid surfaces.[3,4] At the nanoscale, an additional influence of the surface geometry is also known to play a role in governing protein structure and function. In particular, a high degree of surface curvature has been shown to promote increased stability of adsorbed proteins in comparison to macroscopic "flat" supports.[5−10] For example, lysozyme[5] and human carbonic anhydrase[6] retain a greater fraction of their native-like secondary and tertiary structures when adsorbed onto hydrophilic silica nanoparticles (NPs) than on conventional silica surfaces, presumably due to the high degree of surface curvature of the former relative to the latter. A similar phenomenon was also observed for soybean peroxidase upon adsorption onto hydrophobic single-walled carbon nanotubes,[7,8] which resulted in the stabilization of the enzyme under denaturing conditions (e.g., neat organic solvents or high temperatures). Nevertheless, protein stabilization may not be universal. In a recent paper, Roach et al.[9] reported that fibrinogen underwent greater loss of its secondary structure when adsorbed onto smaller silica NPs. Hence, there remains a critical lack of understanding of the influence of nanoscale surface structure on the behaviors of adsorbed proteins.

In the present paper, we demonstrate quantitatively that a model globular protein, ribonuclease A (RNase A, 124 residues, ∼13.7 kDa, 2.2 nm × 2.8 nm × 3.8 nm,[11] pI = 9.4), when bound to spherical silica NPs (4−15 nm in diameter), undergoes significantly different degrees of unfolding in the presence of urea as a function of NP size; more substantial unfolding occurs on the larger particles. This work, we believe, is the first attempt to quantify the impact of NP size and curvature upon the thermodynamic stability of an adsorbed protein.

Ribonuclease A (RNase A) from bovine pancreas and urea were purchased from Sigma (St. Louis, MO) and used without further purification. Silica NPs with nominal mean diameters of 4 and 15 nm were kindly provided by EKA Chemicals, Inc. (Augusta, GA). These particles were dialyzed extensively through 500 MWCO membranes with Millipore water and their concentrations were calculated according to

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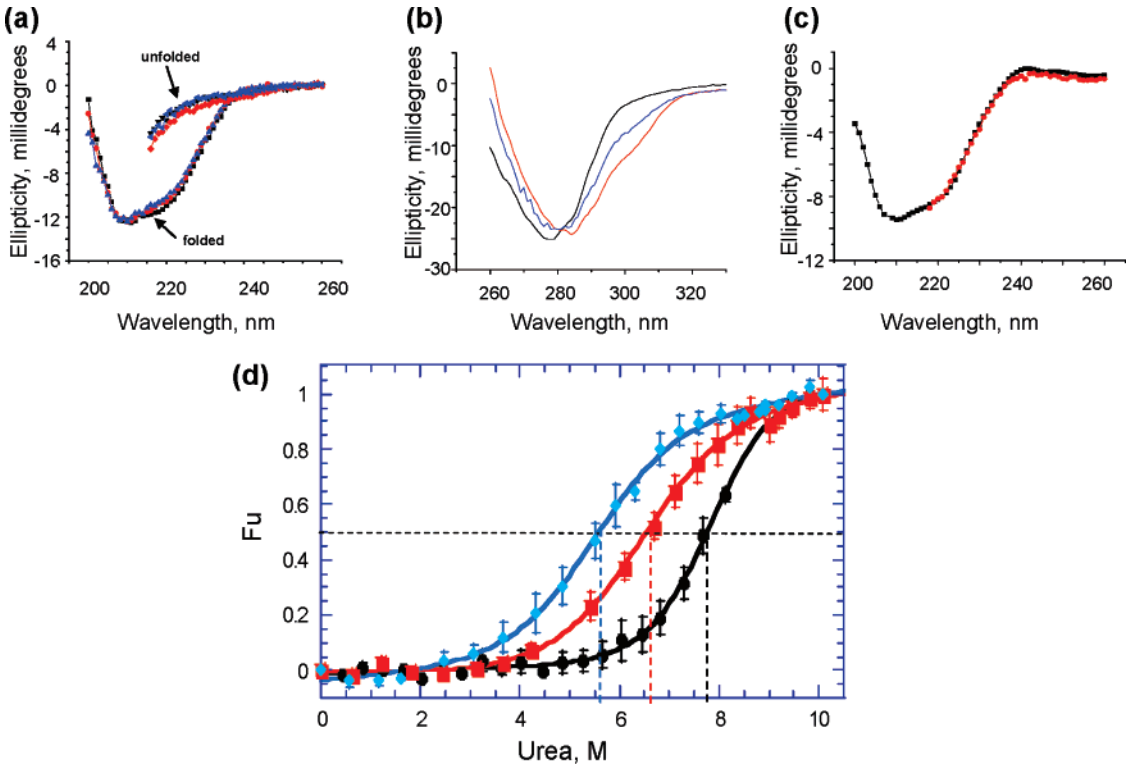

Figure 1. (a) Far-UV CD spectra of free and adsorbed RNase A in both folded and unfolded states. (b) Near-UV CD spectra for free and adsorbed RNase A in the folded state. (c) Refolding of RNase A by 10-fold dilution of the unfolded samples in (a). Only the far-UV CD spectra of RNase A on 4 nm silica nanoparticles are shown; the free and RNase A on 15 nm nanoparticle samples gave similar results. (d) Urea denaturation of free and adsorbed RNase A on silica NPs. For Figure 1a,b,d, black-free RNase A, red-RNase A on 4 nm silica NPs, blue-RNase A on 15 nm silica NPs; for Figure 1c, black-RNase A on 4 nm silica NPs in the folded state; red-RNase A on 4 nm silica NPs after refolding.

particle weight percentages. The spherical silica NPs were characterized by transmission electron microscopy (TEM) and found to have diameters of 3.8 ± 0.6 nm and 15.4 ± 5.0 nm, respectively.

All of the samples were prepared at 20 °C in 2 mM PBS buffer (pH 7.4). Briefly, a stock solution of 10 mg/mL RNase A in 2 mM PBS buffer was mixed thoroughly with silica NPs to reach a final concentration of 0.05 mg/mL RNase A for each sample. A low protein loading (≤5% surface area coverage) was maintained for all of the samples to avoid undesirable protein−protein interactions on the NP surface. Far- and near-UV circular dichroism (CD) analyses were conducted at 20 °C using an OLIS DSM-10 CD instrument (Online Instruments, Bogart, GA) after 18 h of sample incubation at room temperature (long enough for equilibrium to be established[12]). At least three full wavelength scans were taken for each sample, and the spectra were averaged. Quartz cuvettes with optical path lengths of 1 and 0.1 cm were used for the CD measurements.

Far-UV CD analysis of the secondary structure of RNase A revealed no significant loss upon its adsorption onto silica NPs with nominal sizes of either 4 or 15 nm (Figure 1a). Similarly, the near-UV CD signal from the aromatic amino acid residues in RNase A (Figure 1b) revealed only slight red-shifts of the presumed tyrosine peaks for the adsorbed proteins compared with that of the free enzyme. The CD results confirmed that RNase A retained most of its native secondary and tertiary structures when attached onto the silica NP surfaces. To ensure that the NPs were well dispersed and that adsorption of the RNase A on the NPs did not affect their dispersion, TEM observations of the NPs with and without RNase A were carried out. As shown in Figure 2 a−d, there was no change in the dispersion of either the 4 nm (a, b) or 15 nm (c, d) NPs upon adsorption of RNase A.

Having established the retention of RNase A structure when adsorbed onto the well dispersed silica nanoparticles, we proceeded to examine the reversible unfolding of the enzyme while bound to the nanoscale surface. To that end, we incubated the enzyme−silica conjugates in the presence of a series of urea solutions, up to about 9.9 M of the denaturant. At the highest urea concentration, similar to the free RNase A, the adsorbed enzyme also underwent complete reversible denaturation; subsequent 10-fold dilution resulted in the identical CD spectra for all three enzyme preparations when these spectra were compared to their urea-free CD spectra, thereby confirming the reversible folding of both free and adsorbed RNase A upon urea denaturation (Figure 1c).

The reversible folding of adsorbed RNase A further enabled us to study the thermodynamic stability of RNase A that was adsorbed onto the silica NPs using equilibrium urea denaturation. The RNase A−silica NP conjugates were prepared first (0.5 mg/mL RNase A for each sample); urea-induced unfolding was then performed by 10-fold dilution of samples to different concentrations of urea (0−10 M). The ellipticities at 222 nm for all the samples were monitored

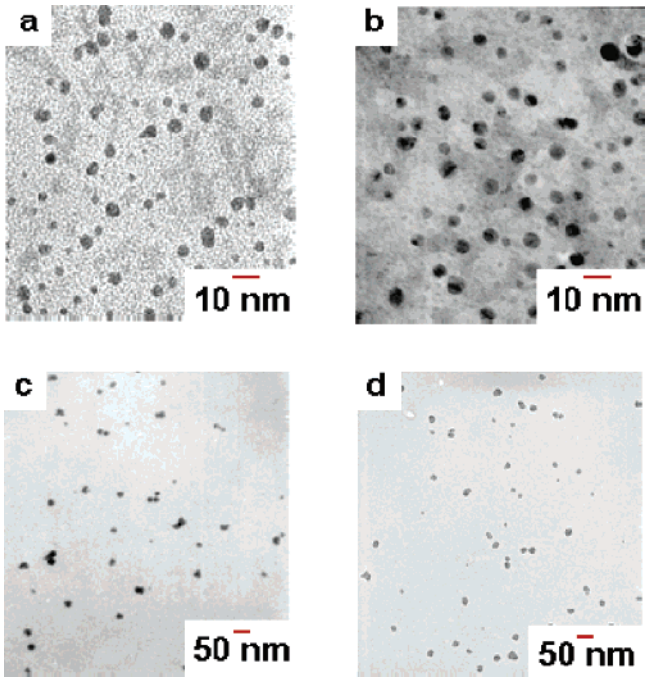

Figure 2. TEM analyses of silica nanoparticles: TEM images of 4 nm particles (a) without RNase A and (b) after attaching RNase A; TEM images of 15 nm particles (c) without RNase A and (d) after attaching RNase A.

by CD after overnight incubation. CD signals for free RNase A samples at different urea concentrations could be examined directly. For the RNase A−NP conjugates, however, there is no direct means of performing solely the CD measurements of adsorbed RNase A, as there is always equilibrium between the adsorbed RNase A and the free form in the solution. To obtain the signals of the adsorbed RNase A, ultracentrifugation (rotor TLA 100.4, Beckman Coulter, Fullerton, CA) was used to separate the unattached proteins from the particles (20 °C and 40 000 rpm for 90 min) after the CD measurement of the mixtures. The CD signal of each supernatant was then obtained afterward. The ellipticities for adsorbed RNase A were calculated by subtracting the corresponding signals for free RNase A in the supernatant from the CD signals for the mixtures. On the basis of the urea denaturation curve of free RNase A under the same condition, the amounts of unbound RNase A at each urea concentration were calculated by assuming that the unbound RNase A behaves the same as free enzyme and also that the CD signal is proportional to the protein concentration in the samples. The CD signals of adsorbed RNase A were then normalized to the same RNase A concentration for each sample. The denaturation curves for adsorbed RNase A were further normalized to the fraction of unfolded RNase A on the nanoparticle surfaces according to the fraction of the signal loss (Figure 1d):[13]

$$(\text{signal}_{\text{tested}} - \text{signal}_{\text{folded}})/(-\text{signal}_{\text{folded}}) \qquad (1)$$

For free RNase A, fitting[14,15] the denaturation data to the classical two-state model[16] resulted in an observed unfolding transition midpoint (C_m) of urea concentration ca. 8 M (Table 1). By plotting the free energy of unfolding ($\Delta G_U = -RT \ln(f_U/f_N)$) as a function of the urea concentration (eq 2), the intrinsic unfolding free energy of RNase A (ΔG_0) in the absence of urea was determined, as was the constant m, which is related to the difference in solvent-accessible surface area between the unfolded and the folded states of the protein.[17]

Table 1. Thermodynamic Parameters Obtained from Equilibrium Urea Unfolding for Free and Adsorbed RNase A and Their Relative Activities

	free RNase A	4 nm	15 nm
ΔG_0, kcal/mol	7.9 (±0.2)	5.4 (±0.3)	3.4 (±0.2)
C_m, M	7.9 (±0.1)	6.7 (±0.3)	5.6 (±0.1)
m, kcal/mol/M	1.0 (±0.1)	0.8 (±0.1)	0.6 (±0.1)
relative activity	100%	90%	66%

$$\Delta G_U = \Delta G_0 - m\cdot[\text{Urea}] \qquad (2)$$

As shown in Figure 1d and Table 1, the stability of RNase A was lower on the silica NPs than in free solution. Interestingly, a clear dependence on particle size was observed, with C_m dropping ca. 15% for the enzyme adsorbed onto 4 nm particles and 30% for the 15 nm particles relative to the free enzyme. Furthermore, the values of ΔG_0 also dropped on the NPs relative to the free enzyme, again with a more significant drop for the 15 nm particles than the 4 nm particles. Such a drop in ΔG_0 relates to a greater propensity of the enzyme to unfold on 15 nm particles than on 4 nm particles.

The m value decreases when RNase A is attached to NPs, with a more substantial decrease on 15 nm particles than on 4 nm NPs (Table 1). The decrease of m indicates that the change in the solvent-accessible surface area during protein folding is smaller than that for adsorbed RNase A owing to its contact with the NP surface. Hence, a larger drop of m on larger silica NPs suggests a greater contact area for RNase A on NPs with larger sizes, which could result in some degree of denaturation. Indeed, on the basis of the assumption that changes in the intrinsic unfolding free energy of RNase A were caused by the unfavorable electrostatic interactions between enzyme and silica, for a single RNase A molecule, we calculate that there are ~1.4 additional charge pair interactions on 15 nm versus 4 nm particles. A greater number of charge pair interactions also suggests a larger area of surface contact of RNase A with larger silica NPs.[18] These results demonstrate that the stability of RNase A is strongly influenced by both adsorption onto silica NPs and the size of the NPs.

We further tested the activity of RNase A based on its RNA-degrading function:[19]

$$\text{RNA} + \text{H}_2\text{O} \xrightarrow{\text{RNase A}} \text{oligonucleotides} \qquad (3)$$

RNase A−NP conjugates were prepared first and incubated at room temperature for 1 day. Yeast RNA solution

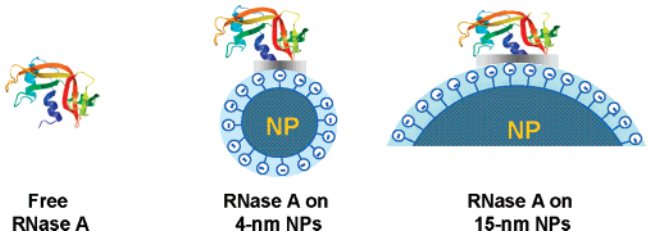

Figure 3. Schematic for the interaction of RNase A with silica NPs with different diameters (4 nm, 15 nm).

was then added to the RNase A sample solutions and mixed thoroughly. UV absorbance at 300 nm for each sample was monitored every 10 s for a total of 12 min. For RNase A−NP conjugates, both activities for enzyme mixtures (including bound and free enzyme in solution) and the free enzyme in the supernatant were determined. The activity for RNase A on the NPs was then calculated and normalized to the percentage of free enzyme activity. Interestingly, the activity of RNase A correlated well with the observed stability changes (Table 1). The results indicated that the enzyme retained 90% of its intrinsic activity on 4 nm silica NPs, while ca. $^1/_3$ of its activity was lost when attached onto 15 nm NP surfaces.

To explain the observed changes in RNase A stability and activity as a result of its adsorption onto silica NPs of different sizes, we use a simple model depicted in Figure 3. The model is consistent with other protein−silica NP systems.[5,6,9] The primary interaction between positively charged residues on RNase A and the negatively charged silica surface is governed by Coulombic interactions, which strongly depend on the surface area of contact between the protein and silica and also the electrostatic potential of the nanoparticle surface. A larger surface area of contact will strengthen the protein−nanoparticle interaction, as will a higher surface potential. For larger nanoparticles (with smaller curvature), the contact area between the protein and silica is greater than that for smaller nanoparticles.[6] The surface potential for larger nanoparticles is also higher.[5] These two effects lead to stronger interactions between RNase A molecules and the larger nanoparticles and result in a greater perturbation of the stability of adsorbed RNase A on 15 nm versus 4 nm silica NPs.

At pH 7.4, there is a dominant positively charged domain at one end of the RNase A molecule (PDB code 2AAS),[20] along the longest axis of the molecule. When RNase A is adsorbed onto silica NP surfaces, the enzyme is, therefore, likely to be attached to the negatively charged silica with its positive domain in contact with the particle surface. Moreover, the active site residues His 12 and His 119 sit close to this positively charged domain. A larger contact area of RNase A with larger NPs could cause the active sites to become partially blocked and therefore result in the decreased activity. The drop of the activity of RNase A is consistent with the model shown in Figure 3.

During the latter stages of this study, we observed that the 4 nm silica NPs appeared to agglomerate into larger nonspherical particles after several additional months of storage. These agglomerates were characterized by TEM

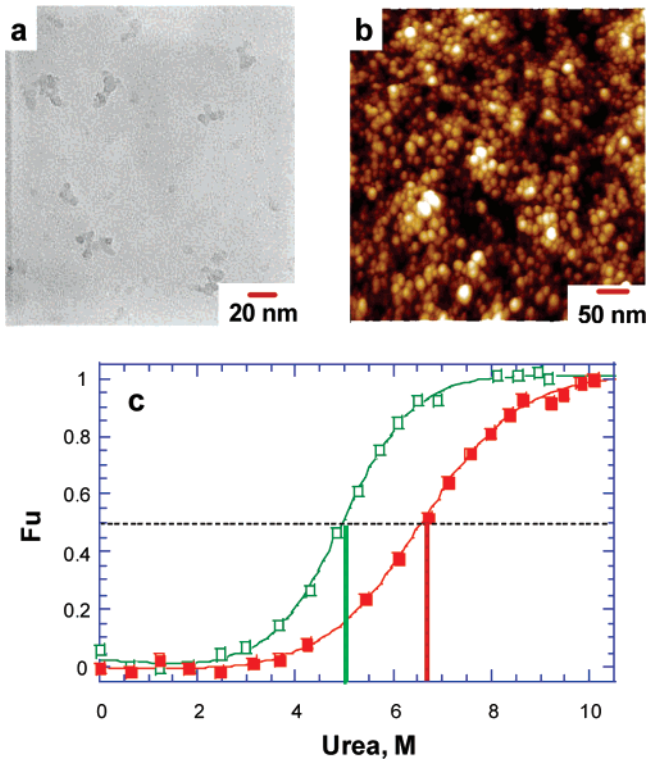

Figure 4. TEM and AFM analyses of agglomerated silica nanoparticles and the stability of RNase A on them: (a) TEM image of the agglomerated 4 nm particles. (The imaging of these agglomerated particles is difficult, which might be due to the melting[21] of the nonconductive agglomerates of the silica NPs under the TEM.) (b) AFM topographic image of agglomerated 4 nm NPs. (From a size distribution analyses of 200 agglomerated silica nanoparticles, the average size of the agglomerates is 8.1 ± 2.3 nm. Using a model based on both spherical AFM tip and particle agglomerates, the actual equivalent diameter of the agglomerates (2Δ) is given by the equation:[22] $2\Delta = 2\sqrt{r(2R-r)}$ where R represents the apex radius of the AFM tip and r represents the measured radius of the particle agglomerates. For the measured radius of $r = 4.1 \pm 1.2$ nm and an apex radius of the high aspect ratio Si tips used for the measurements of $R = 5.0 \pm 2.0$ nm, the actual equivalent diameter of particle agglomerates is 9.8 ± 3.6 nm.) (c) Stability of RNase A on 4 nm silica nanoparticles (red-RNase A on well dispersed primary 4 nm silica nanoparticles; green-RNase A on agglomerated 4 nm silica nanoparticles).

(Figure 4a) and confirmed by atomic force microscopy (AFM) (Figure 4b) to have approximate spherical-equivalent diameters of 10 nm (see Figure 4 caption). We took this opportunity to further investigate the particle-size dependent stability of adsorbed RNase A. Interestingly, the stability study indicated that RNase A was less stable on these larger agglomerated particles than on the original well dispersed 4 nm particles (Figure 4c). The unfolding transition midpoint C_m for RNase A that adsorbed onto the original well dispersed 4 nm silica NPs is 6.7 M of urea, while it decreases to 5.0 M of urea for RNase A on the agglomerated particles. Consequently, the unfolding free energy of RNase A (ΔG_0) also drops (5.4 kcal/mol for RNase A on the original well dispersed 4 nm NPs and 3.6 kcal/mol for that on the agglomerated NPs). These results are also consistent with the model shown in Figure 3. Compared to the surface contact area on the original 4 nm particles, the surface contact

area between the enzyme and the large nonspherical agglomerated particle surface would increase and the stability of RNase A therefore decreases. The stability of RNase A on the agglomerated NPs, however, is close to what was observed for RNase A on 15 nm particles ($\Delta G_0 = 3.4$ kcal/mol), although the size of the agglomerated particles appears to be smaller than the 15 nm NPs. This phenomenon may be due to the rougher surface and/or irregular shapes of the agglomerated particles. The different morphology of the agglomerated particles might also contribute to the destabilization of the adsorbed RNase A. Clearly, however, considerable further detailed investigation, beyond the scope of present work, will be needed to elucidate the correlation between nanoscale surface roughness, particle morphology, and protein adsorption and resulting structure changes.

In conclusion, the present study demonstrates that the unfolding of RNase A on spherical silica NPs is strongly affected by the size, and hence surface curvature, of the support. The enzyme is less stable on NP surfaces than in free solution, and the stability is decreased further on larger particles with smaller surface curvature. Thus, for the first time, it is quantitatively shown that a physical property at the nanoscale (e.g., particle size or surface curvature) influences the stability and unfolding of adsorbed proteins. These results may find particular relevance in understanding protein functions in both unnatural nanoscale regimes (e.g., hierarchical hybrid material−biomolecule assemblies) and natural assemblies within cells (e.g., cell and organelle membranes).

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